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13. ABSTRACT *(Maximum 200*

The objectives of the research program are to study the structure-immunogenicity relationships of a hypoglycosylated human tumor-specific mucin common to breast and other adenocarcinomas. Hypo-glycosylation of breast mucin leads to exposure of a tumor-specific epitope (TSE). The structural and immunogenic properties of the TSE are being examined using synthetic mucin peptides and recombinant mucin proteins that contain the TSE and/or mutations in potential glycosylation sites surrounding the TSE. One TSE is the pentapeptide PDTRP of the 20 amino acid mucin tandem repeat sequence PDTRPAPGSTAPPAHGVTSA. Immunogenic cross-reactivity with tumor-specific monoclonal antibodies show that antigenicity is maximized with the 40 amino acid MUC1-mtr$_2$. By contrast, the MUC1-mtr$_3$ elicited a 9-fold better cellular response compared to either single or double tandem repeat peptides. The single, double and triple tandem repeat peptides show few elements of preferred conformation, based on ^{1}H-NMR spectroscopy. A model of the distribution of the oligosaccharide side chains along the mucin core protein was developed showing that the carbohydrates may not surround the protein in a uniform "coating". Thus, regions of the core protein are exposed under normal circumstances, yet tumor-specific epitopes remain masked by the sugars in the non-malignant cells. Further understanding of the structure-immunogenicity relationships of tumor-specific immunogens is essential for maximizing the use of synthetic peptide immunogens and tumor-specific cells as a potential adoptive immunotherapy for patients with cancer, and the development of a potential vaccine.

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K E Eckert 9/11/97

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Introduction. Immunotherapy is a state-of-the-art approach to augment existing cancer therapies of radiation, chemotherapy and surgery. Immunotherapy takes advantage of the specificity of the immune system to recognize and rid the body of tumor cells. Immune cells have been identified which recognize and kill tumor cells in a specific manner [e.g. tumor-infiltrating lymphocytes (TIL); [1-3], as well as in a non-specific manner [e.g. lymphokine activated killer (LAK) cells and natural killer (NK) cells]. Pertinent to this research program, T-cells (4-6) and B-cells (7) specific for breast, ovarian and pancreatic adenocarcinomas have been described. These observations demonstrate that the body does mount an immune response against several adenocarcinomas.

Tumor proteins or tumor antigens associated with the development or expression of the malignant cell often render these cells immunogenic. Pertinent to this proposal is normal self-proteins that are aberrantly expressed or post-translationally modified on the tumor cells compared to the normal cells. Polymorphic epithelial mucin (8; mucin for this report) is a normal protein that is aberrantly expressed on the tumor cells. As such, mucin becomes a tumor-specific protein.

Mucin is the major glycoprotein of mucous secretions and is normally confined to the luminal surface of the glandular epithelial cells (8, 9). The core protein is heavily glycosylated with carbohydrate accounting for up to 80% of the glycoprotein mass (8, 9); the sugars O-linked to the protein through serine and threonine (8). The entire mucin core protein has been cloned and sequenced (8). To date, at least 7 different mucin proteins have been described. The hallmark of mucin is tandem repeating sequences. MUC1 is the mucin associated with breast, ovarian, pancreatic and several other adenocarcinomas. MUC1 has the 20 amino acid repeating sequence of P^1DTRPAPGST^{10}APPAHGVTSA^{20} (MUC1-mucin tandem repeat; i.e., MUC1-mtr_1). Mucins are structurally polymorphic and generally contain between 40-100 tandem repeat sequences.

The structure of the MUC1-mtr_n, where n=1-3, has been partially characterized based on NMR, hydropathicity and structure prediction calculations (10-13). Viscosity measurements suggested that the structure is rod-shaped (12). Residues P^1DTRP^5 are suggested to be poly-proline β-turn elements described as "knob-like" protrusions (11-13). It is the poly-proline β-turn regions of the core protein which are suggested to be within the tumor-specific epitope (12). This model is intriguing in that the positions of all of the potential glycosylation sites surround the tumor-specific epitope, which is contained in the β-turn regions. Thus, glycosylation of any, or all, of these residues may result in altered immunogenicity of the tumor-specific epitope.

The extensive branching of the carbohydrate side chains of the mucin glycoprotein inhibits the tumor-specific antigenicity and immunogenicity of mucin. Cancer-associated mucins are aberrantly glycosylated in that the carbohydrate side chains are shorter than those of the mucin produced by normal cells (8). These mucins have unique antigenic epitopes exposed on the core protein which are masked in the fully glycosylated form (8). Other non-tumor-specific epitopes are expressed in both the normal and tumor-associated mucins (8). The presence of tumor-specific epitopes is evidenced by the development of many monoclonal antibodies (mAb) that recognize tumor-specific mucins and apo-mucin, but not the fully glycosylated molecule (8). The epitope recognized by a representative mAb, SM3, is the sequence P^1-P^5 in the tandem repeat sequence (4). This epitope was identified by competition of antibody binding to tumor-specific mucin by synthetic peptides of the mtr (8).

Human cytotoxic T lymphocyte (CTL) cell lines from breast (4, 6), ovarian (5, 6), and pancreatic (4) adenocarcinomas have also been described which recognize mucin-expressing cell lines. One cell line, WD, was derived from the tumor-draining lymph node cells from a patient with pancreatic adenocarcinoma by continuously stimulating the cells with allogeneic pancreatic tumor cell lines. WD has a $CD3^+$, $CD4^-$, $CD8^+$ phenotype. Pancreatic mtr sequences are identical to the breast mtr (8). This explains why CTL generated against pancreatic carcinoma recognize breast carcinoma (4). The WD CTL did not recognize colon cancer cell lines, as expected, since the primary sequence of colon mucin expressed by cell lines differs from the sequence of the breast mucin peptide (8). These results show specificity of the immune recognition. Also, immunohistochemical staining and Northern blot analysis of pancreatic and breast tumor cell lines with SM3 showed that the cell lines lysed by WD CTL express mucin epitope recognized by the monoclonal antibody (4). Fresh colon cancer tissue expressed the same mucin as breast and pancreatic cancers, i.e. MUC1 (14). The colon cancer cell lines do not express MUC1, but express other genes of the mucin family (MUC3 & MUC4). In addition, renal, gastric, lung and ovarian cells, express MUC1 (8, 14). This supports the hypothesis that mucin is a common tumor antigen on multiple adenocarcinomas.

Target cell recognition by these CTL lines is Human Leukocyte Antigen (HLA)-non-restricted. This was shown by the ability of mucin-specific CTL cell lines to recognize and kill non-HLA-matched tumor target cell lines. One explanation for this is that mucin, due to its multiple tandem repeat sequences, is a multivalent antigen. Multiple epitopes on the tumor cell surface not associated with HLA are postulated to bind to, and cross-link, the T cell receptor (TCR), thus leading to CTL activation and target killing (4, 15, 16). Another explanation for the lack of HLA-restriction is that adenocarcinoma cells do not express HLA-class I molecules (17). By contrast, CTL recognition of target cells bearing a single tandem repeat of mucin is HLA-restricted (18). Barnd et al. (4) and Ioannides et al. (6) postulated that the mucin epitope bound to HLA class I (T-cell epitope) is included in, or adjacent to, the same epitope that is recognized by the SM3 monoclonal antibody (8). This was based on the observation that SM3 bound to cancer cells expressing mucin inhibited the recognition and lysis of the target cell by CTL. The CTL epitope was narrowed to residues P^1-T^{10} that includes the sequence recognized by SM3 (5). Antibodies to sequences outside these 10 amino acids had no inhibitory effect on the lysis of mucin-bearing adenocarcinomas by CTL (5). This suggests that the monoclonal antibody binding site and the T-cell epitope are the same, or near one another. However, it was not clear whether the inhibition of cytolytic activity was due to steric hindrance as a result of antibody binding. The exact sequence of the HLA-bound tumor-specific mucin T cell epitope remains to be determined.

We have used an algorithm (19) to predict the potential HLA class I-bound mucin peptide. This work showed for both the MUC1-mtr_1 and (T3N)-MUC1-mtr_1, a peptide with a mutation in a potential glycosylation site within the tumor-specific mAb site which also elicited tumor-specific T cells (5), the nonamer $S^9TAPPAHGV^{17}$ has the highest predictive value for the potential T cell epitope. This suggests that a mutation in a potential glycosylation site within the mAb tumor-specific epitope does not effect the potential T cell epitope. Experimental evidence has also been published suggesting that this nonamer may be a T-cell epitope (20). This peptide bound to HLA-A1, -A2.1, -A.3 and -A11. The affinity of HLA-A11 for this peptide was close to that found for HLA-bound non-mucin peptides. Since this peptide is "outside" the range of the T cell epitope suggested by Ioannides et al. (5), it is

reasonable to conclude that the inhibition of cytolytic activity by SM3 was due to steric hindrance and not to masking of the epitope by the antibody. These results also suggest HLA class I-restricted T cell antigen recognition of tumor-specific mucin.

Multiple approaches have been used to generate mucin tumor-specific CTL. These methods have involved the use of hypoglycosylated mucin, obtained from autologous and allogeneic tumor cells or expressed as a recombinant protein, as the immunogen. For example, mucin has been expressed in a mammalian system using phenyl-N-acetyl-α-galactosaminide (21), an inhibitor of glycosyl transferases involved in the early chain elongation of O-linked oligosaccharides. However, the initial N-acetylgalactosamine (GalNAc) still modifies the core protein (21). Personal communications (22, 23) have stated that SM3 binds to MUC1 modified by a single GalNAc, but no published evidence to supports this hypothesis. Also, Finn and co-workers (24) have used a truncated mucin, suggested to be non-glycosylated using antibodies claimed to recognize glycosylated vs. non-glycosylated mucin; however, these mucins were not biochemically proven to be non-glycosylated. Therefore, this raises questions of the utility of this approach for generating mucin-specific CTL. We address the issue of obtaining a reproducibly non-glycosylated of mucin immunogens below and the structure-immunogenicity relationship of tumor-associated MUC1.

Body. Antigenicity and immunogenicity of synthetic and recombinant mucin peptides. Our approach to the generation of adenocarcinoma-specific CTL from the peripheral blood mononuclear cells (PBMC) of patients with breast or ovarian cancer is to stimulate these lymphocytes with synthetic mucin peptides. Since glycosylation masks mucin tumor-specific epitopes, the synthetic nature of these peptides ensures reproducible non-glycosylation of the immunogen and unmasking of core protein tumor-specific epitopes. The synthetic peptide immunogens, ranging from five to 60 amino acids in length, are described in Fig. 1. Additionally, we have constructed a synthetic gene encoding five tandem repeats of the tumor-specific epitope of human mucin was designed for efficient cloning and expression in *E. coli*. The synthetic gene was cloned in the correct reading frame into the maltose-binding protein (MBP)-fusion expression vector pMAL-p2. Bacterial clones containing the mucin synthetic gene insert produced a protein that bound to amylose resin, verifying the presence of the MBP moiety of the fusion protein, and was reactive with monoclonal antibodies which are specific for human mucin, verifying the presence of the mtr_5 polypeptide. Thus, this fusion protein is consistent with the intended recombinant fusion protein, MBP-mtr_5. Furthermore, the fusion protein produced represents a significant fraction of the cellular protein, and is not heavily degraded. This result represents a major advance over the expression of a previously published seven tandem repeat-containing fusion protein which was a human gene expressed in bacteria that was heavily degraded (25). MBP-mtr_5 was purified by affinity chromatography on amylose resin, and for immunological studies. However, in order to perform structural studies, the mucin peptide must be released from its fusion partner. This fusion protein was designed by including a protease Factor Xa site to link the N-terminus of the mucin polypeptide to the C-terminus of MBP. Although the mucin tandem repeat polypeptide does not contain the canonical Factor Xa cleavage site, this polypeptide is digested by the protease, resulting in the loss of the mucin peptide, based on immunocrossreactivity with the mucin core protein-specific mAb HMFG-2 (26). Although we do not fully understand the reason for this outcome, it is possible that Factor Xa has "star" activity on the mucin protein. To overcome

this problem, we are in the process of subcloning the mucin cDNA into a fusion protein system with a genenase cleavage site; genenase does not cleave the MBP-mtr_5, and thus should be a suitable expression system for this polypeptide.

All synthetic peptide and bacterial-expressed immunogens contain a monoclonal antibody (mAb) tumor-specific epitope, PDTRP, and are antigenic (Fig. 1). Residues flanking the tumor-specific epitope profoundly influence the antigenicity of the peptides; the presence of amino acid residues to the N-terminus of the epitope appear to be more important to the antigenicity of the peptides than do residues to the C-terminus. Fig. 1 shows that the MUC1-mtr_1 is not recognized strongly by the mAb, HMFG-2, but MUC1-mtr_2 cross-reacts with this mAb. Increasing the length of the mucin peptide to three tandem repeats (MUC1-mtr_3) or five tandem repeats (MBP-mtr_5; not shown) does not enhance the antigenicity of the molecule. This suggests that there are no cooperative structural contributions that occur by increasing the length of the polypeptide. Peptide p609, which contains the mAb epitope in the center of the peptide, cross-reacted with the mAb, but to a lesser extent than did peptide p610, which contains the mAb epitope near the C-terminus of the peptide. Peptide p610 was recognized by HMFG-2 to approximately the same extent as the MUC1-mtr_2. Since the same molar equivalent of each peptide was assayed, we concluded that residues flanking the tumor-specific mAb epitope influences the antigenicity of the molecule, but there are not significant cooperative conformational features added by increasing the number of tandem repeats. Maximal antigenicity is achieved with p610 and the 40 amino acid, double tandem repeat peptide, MUC1-mtr_2. NMR conformational studies are in progress to fully understand the structural basis of this result.

The cellular immune response to the MUC1 mucin peptides differs from the antigenicity of the peptides. Whereas MUC1-mtr_1 reacts poorly with the tumor-specific mAb (Fig. 1), stimulation of PBMC from patients with either breast or ovarian adenocarcinoma by MUC1-mtr_1 + IL2 results in the reproducible expansion of adenocarcinoma-specific cytolytic T cells that are not restricted by HLA for target cell recognition and lysis (6). Peptide stimulation induced an oligoclonal expansion of cytolytic T cells with a 5-10-fold greater cytotoxicity against the MUC1-mucin-expressing breast cancer cell line, MCF-7, compared with PBMC stimulated by either anti-CD3+IL2, or IL2 alone. These cultures were phenotyped and found to be a mixture of $CD4^+$ and $CD8^+$ T cells; no significant expansion ($<$ 2%) of natural killer (NK) cells was observed (1). Thus, peptide-stimulated T cells showed antigen specific activation.

We have expanded our results with MUC1-mtr_1 to compare the effect of tandem repeat length on the stimulation of tumor-specific CTL from breast cancer patient PBMC. Doubling the length of the mucin peptide immunogen, MUC1-mtr_2, does not result in better expansion of tumor-specific CTL when stimulated with the same molar equivalent of tumor-specific epitopes of MUC1-mtr_1 (not shown). By contrast to the single and double tandem repeat peptides, incubation of human PBMC with the 60 amino acid MUC1-mtr_3 in the presence of IL-2 generated cytolytic lymphocytes from the PBMC from a donor with breast cancer that recognized the MUC1-mucin-expressing breast tumor target line MCF-7 (Fig. 2A). Non-stimulated (Fig. 2C), or IL-2-stimulated (Fig. 2B), PBMC do not exhibit cytolytic activity against this target cell. This peptide stimulated the expansion of tumor-specific T cells from non-cytolytic PBMC from two donors with breast cancer and one with ovarian cancer ~ nine times better than the MUC1-mtr_1 or MUC1-mtr_2 (not shown). This cell line also exhibited

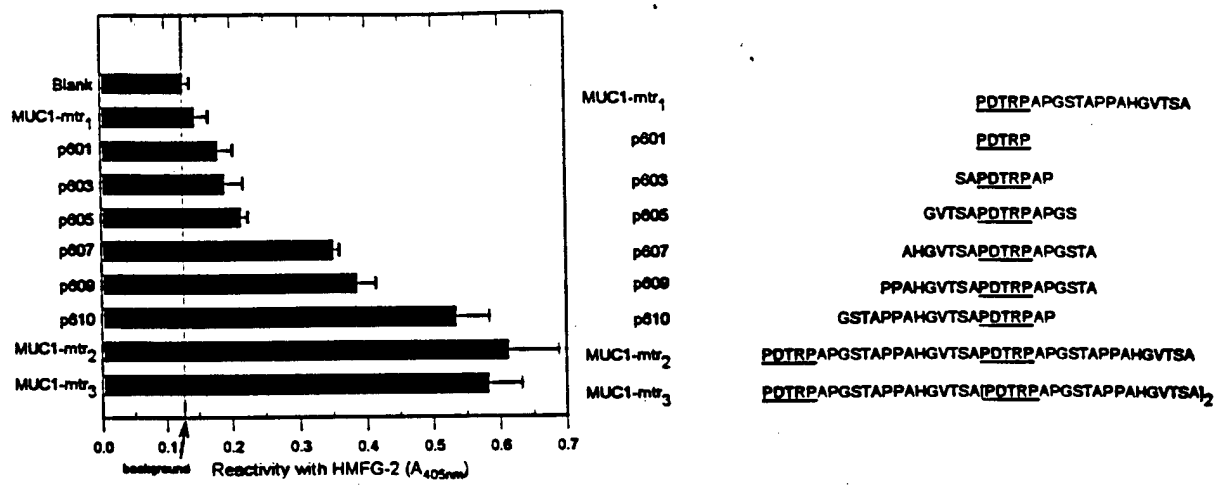


Figure 1. Antigenicity of MUC1-mucin peptides. All wells were coated with the same molar equivalent of the tumor-specific epitope PDTRP (bolded and underlined in right panel). Antigenicity was determined by reactivity with the tumor-specific mAb HMFG-2 by ELISA.

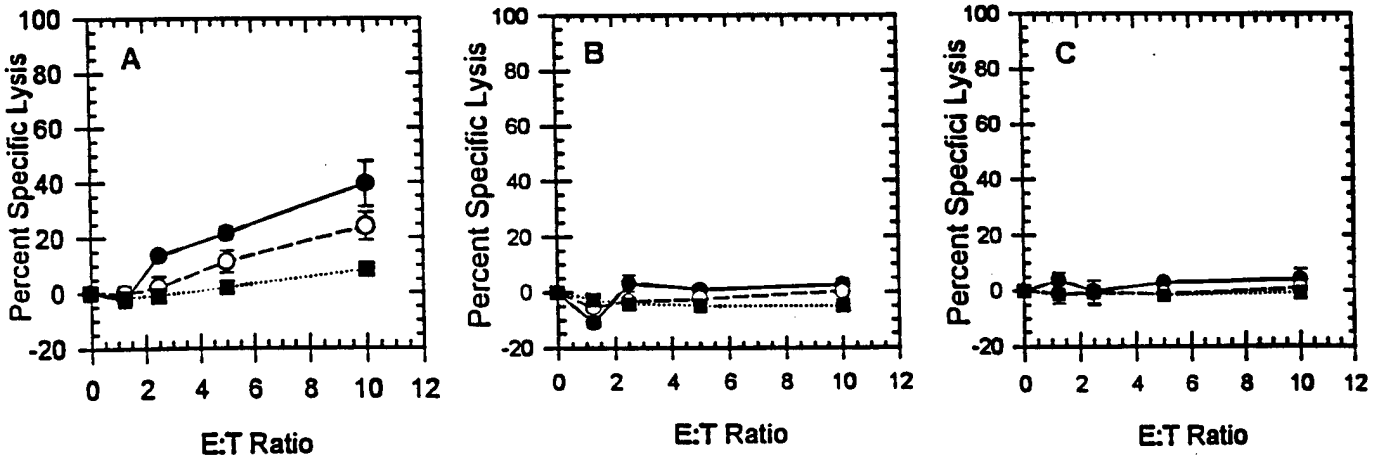


Figure 2. Representative experiment showing tumor-specific cytolytic activity of PBMC from a single donor with breast adenocarcinoma following MUC1-mtr$_3$ peptide-stimulation. Bulk CTL were generated by stimulation of PBMC for 28 days with (A) MUC1-mtr$_3$+ 100 IU IL2 or (B) 100 IU IL2 alone as we described previously for MUC1-mtr$_1$ (1). (C) Unstimulated PBMC were frozen on day 0 (with no cytolytic activity) and thawed on day 28 and assayed in parallel with IL2- and peptide-stimulated cells. Cytolytic activity was determined by ^{51}Cr-release assay (1) on day 28 of culture with effector cells against target cells: (●) MCF-7 breast tumor cell line; (○) K562, a NK cell target; and, (■), Raji, a LAK cell target.

cytolytic activity (~ 20%) against the NK target, K562, but not against the LAK target, Raji (< 10%), raising the possibility that NK cells were expanded from peptide stimulation. Thus, the phenotype of these cells is currently being determined, and lymphocytes will be sorted by CD markers (e.g., $CD4^+$, $CD8^+$, $CD56^+$) and assayed against breast and other adenocarcinoma cell lines expressing hypoglycosylated MUC1-mucin for tumor-specific cytolytic activity to identify the cells responsible for the cytolytic activity. Despite the phenotype of this bulk cell line, tumor-specific killer lymphocytes are expanded by this immunogenic mucin peptide.

Conformation of MUC1-mtr_1 peptides. We are also studying the conformational preferences of human mucin (Ribeiro, AA, Spicer, LD, Wright, SE & Dombrowski, KE, manuscript in preparation). As we have reported previously, we have made the sequential proton assignments for the MUC1-mtr_1 (Fig. 3A) and (T3N)-MUC1-mtr_1 (not shown) in water based on COSY, TOCSY and NOESY ^{1}H-NMR spectroscopy. We have observed reasonable dispersion of backbone NH and α-CH resonances. The temperature dependence of the backbone NH resonances was also examined over the range of 0-35 °C. Temperature coefficients were of intermediate value and show little evidence for a single stable secondary structure (not shown). However, three residues (R^4, S^9, and G^{16}) show less temperature dependence. This suggests that these residues remain in a more stable conformation relative to the other amino acids. Based on NMR studies in d^6-DMSO (12) a β-turn was proposed as a major conformational feature of native mucin peptide. Initial NMR studies in aqueous phosphate buffer at pH 6.8 (11, 12), the single native mucin peptide yielded broad, overlapped NH signals, and these workers concluded that the native repeat peptide is largely disordered in solution. More recently, better-resolved 2D spectra (12) also indicate a largely unordered structure with evidence for "knob-like" domains.

Our data obtained at pH 4 show good dispersion of backbone NH and α-CH resonances in both the native and mutant peptides. This supports the case for the presence of elements of secondary structure. Our data also provide clear evidence that no large global changes occur upon substitution of the uncharged polar Thr side chain [-CHOH-CH_3] with the uncharged polar Asn side chain [-CH_2-$CONH_2$]. Only local shift changes are seen: the T^3 NH is replaced by the N^3 NH and concomitantly the R^4 NH moves upfield. The remaining amino acid resonances have similar shifts and similar temperature behavior. Since there is little evidence for a single stable secondary structure, it may well be that more than one tandem repeat sequence is needed to stabilize a more ordered polypeptide. However, a sufficient population of preferred peptide conformers may exist in a single tandem repeat to elicit the immunogenic response.

While our detailed NMR efforts have focussed on the single 20 amino acid tandem repeat peptide, MUC1-mtr_1, we have obtained our first NMR observations for the 40 amino acid, double tandem repeat peptide, MUC1-mtr_2 and the 60 amino acid MUC1-mtr_3. The His^{15} and His^{35} C2H proton signals are found to be equivalent in chemical shift in MUC1-mtr_2, MUC1-mtr_3 and to resonate at the same value as the His^{15} in MUC1-mtr_1. Since these protons are not exchangeable with solvent water protons, we can compare the intensities of the NH signals of MUC1-mtr_1 and MUC1-mtr_2 by scaling relative to the number of histidine protons. Fig. 3 shows the NH resonances at 5 and 35°C, respectively. The bulk of the NH resonances at both temperatures are found to behave similarly to the His C2H signals, i.e., the shifts of

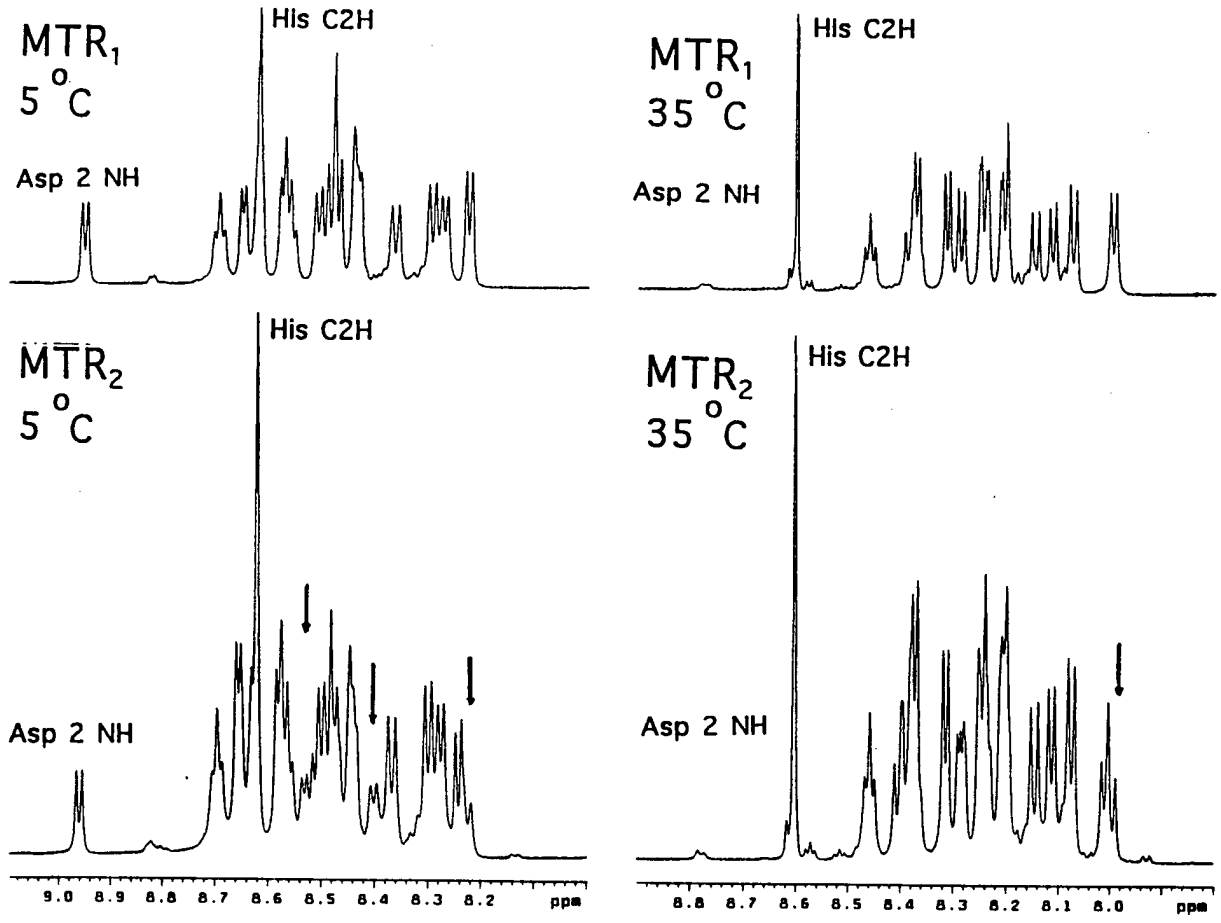


Figure 3. Comparison of the amide NH region of MUC1-mtr_1 (top panel) and MUC1-mtr_2 (bottom panel). NMR spectra were obtained in water at 5 °C and 35 °C.

the second repeat are coincident with the shifts of the first repeat unit. However, a closer examination of the 5°C spectra reveals three new NH resonances in MUC1-mtr_2 not observed in MUC1-mtr_1, and that the resolved Asp^2 NH signal 8.96 ppm integrates to only one proton in both the single and double tandem repeat peptides. This implies that Asp^2 and Asp^{22} of MUC1-mtr_2 are not coincident in chemical shift. Moreover, at 35°C, loss of NH intensity due to exchange with solvent is apparent mainly at the Asp^2 NH in both mucin peptides.

Recent NMR observations have now been obtained with the MUC1-mtr_3. Fig. 4 compares the spectra of the single (Fig. 4, *upper panel*), double (Fig. 4, *middle panel*) and triple (Fig. 4, *lower panel*) mucin tandem repeat peptides at pH 4 and 5°C. The spectra were normalized to the His C2H at full scale. The Asp^2 NH proton (8.9 ppm) shows a ratio of 3:2:1 (monomer: dimer: trimer). MUC1-mtr_2 and MUC1-mtr_3 have not previously been studied at this detailed level and with this excellent dispersion of backbone protons, and further work should provide new insights into the conformational preferences of these tandem repeats.

Surface Analysis of Native and Deglycosylated Mucin. X-ray photoelectron spectroscopy (XPS) is a surface sensitive analytical technique that measures the binding energy of electrons in atoms and molecules. The binding energy can be related to the molecular bonding or oxidation state of an element in the outermost layer of a material (<6 nm). Thus, XPS is able to identify chemical species present on the surface of a molecule. The quantitative XPS results of C, O and N for amino acids, simple carbohydrates and peptides of human mucin have been previously determined (27).

One of the goals of the proposed research is to correlate the amount of carbohydrate coating (e.g., thickness) with immunological response for human mucin. Mucin from human breast milk was chosen to model the fully glycosylated mucin. The breast milk mucin was purified according to published procedure (28). Based on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry, the mucin sample was homogeneous and free from low molecular weight contaminating proteins. We previously reported a model for MUC1 mucin from breast milk where the carbohydrate side chains do not completely "coat" the core protein in the native mucin molecule (29). We have continued to refine this model, and have begun to examine the sensitivity of this analytical method to determine whether we can determine which residues may be exposed to the aqueous environment, and thus, not masked by the protecting carbohydrate. This issue becomes of importance to determine whether the immune system may be tolerized to regions of the core protein that may be normally exposed, and thus, expansion of tumor-specific CTL may be more difficult than anticipated. Thus, by understanding which residues are exposed, we may be able to better develop better tumor-specific immunogens.

The N1s XPS spectra of MUC1 mucin from three sources are shown: synthetic MUC1-mtr_1 (Fig. 5, *upper panel*), MUC1-mtr_3 (Fig. 5, *middle panel*) and milk mucin purified from human breast milk (Fig. 5, *lower panel*). All three spectra show evidence for more than one chemical bonding state of nitrogen. That is, the peak noted at ~ 400 eV is characteristic of nitrogen found in the amide bonds of the mucin protein, whereas the structure extending from approximately 401 to 403 eV is characteristic of nitrogen with a positive charge. This type of structure would result from the presence of a positive charge. This type of structure would result from the presence of N-terminus zwitterions or more likely in the guanidinium structure

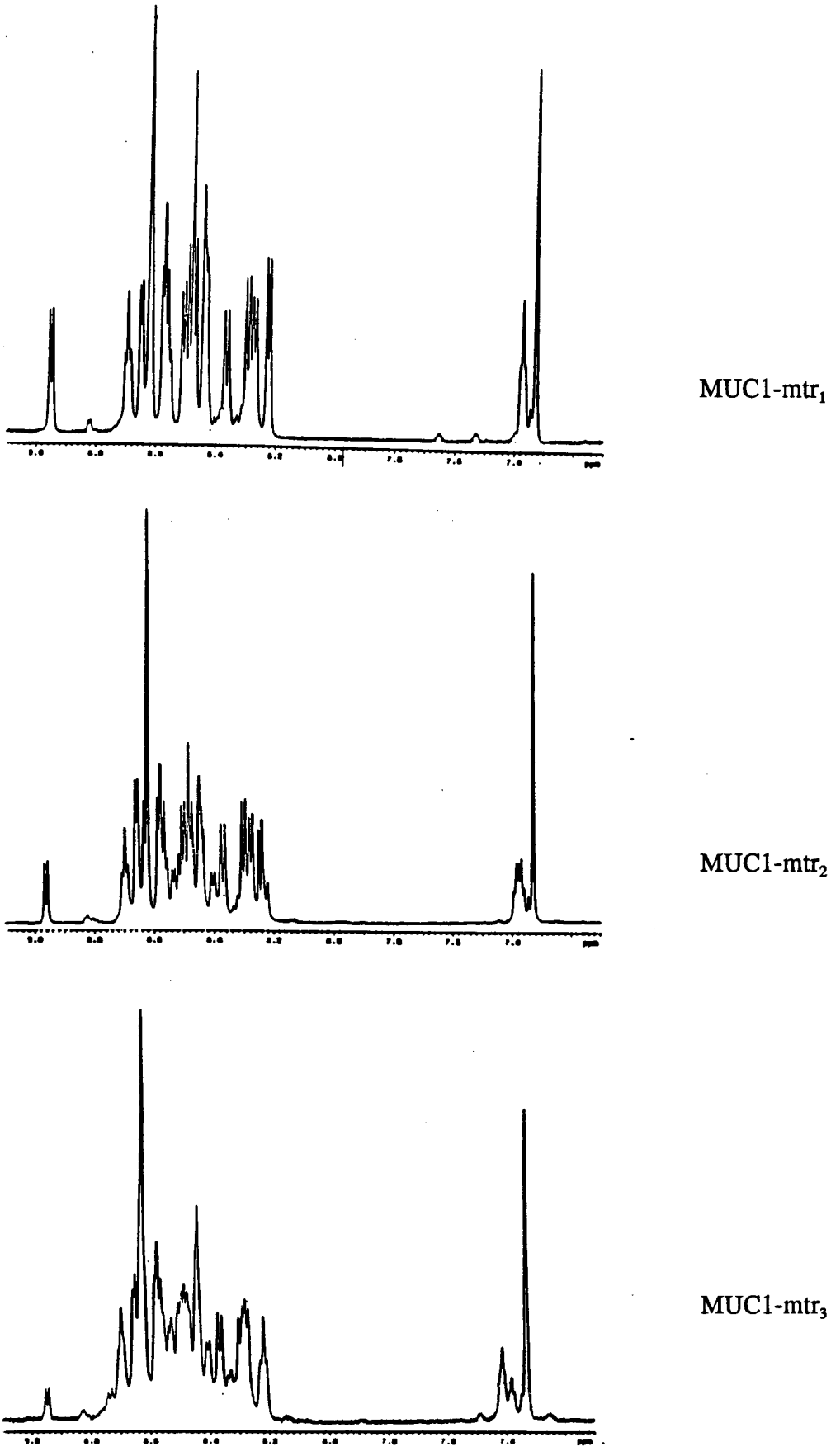
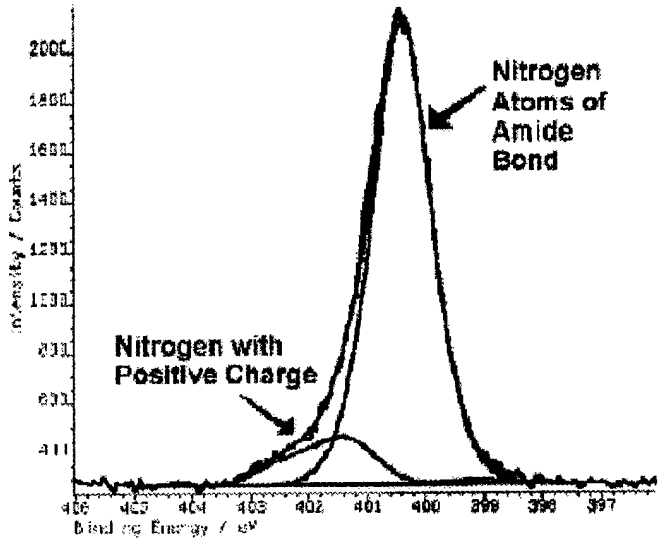


Figure 4. Comparison of the ^{1}H-NMR spectra for the single (upper panel), double (middle panel) and triple (lower panel) MUC1 mucin polypeptides.

A



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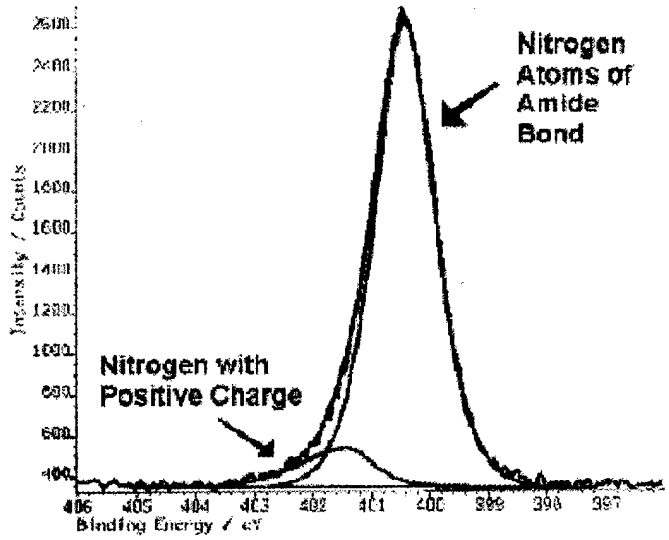

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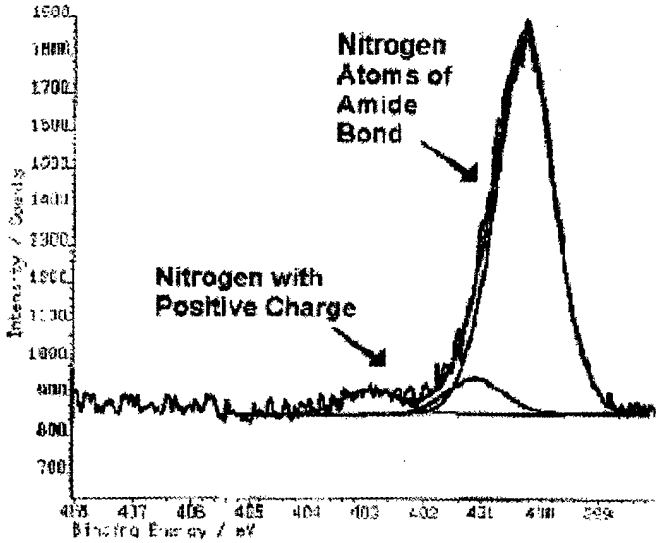

Figure 5. N 1s XPS spectra of (A) MUC1-mtr$_1$, (B) MUC1-mtr$_3$ and (C) purified breast milk MUC1 mucin. Jagged red lines are raw data; smooth lines are the deconvoluted peaks indicated

of the arginine or the imidazole ring of histidine in the mucin peptide. This positively charged nitrogen is a few percent of the total nitrogen signal. Both arginine and histidine are considered to be part of the mAb and CTL epitopes, respectively, therefore, these positive charge nitrogen ions may play a role in the immunogenic activity of the mucin. This effect will be evaluated in the future.

Conclusions. The goal of this work is to understand the structure-immunogenicity relationships of the tumor-specific mucin. In the first three years of this work we have used synthetic mucin peptide immunogens, up to 60 amino acids long (MUC1-mtr$_3$), with both native sequences and with mutations in potential glycosylation sites. Because the product yield of automated solid-phase protein decreases substantially above 60 amino acids (e.g. three tandem repeats of mucin), we have designed and expressed from bacteria a recombinant mucin polypeptide containing five tandem repeat elements in a non-degraded form. We initially proposed to construct and express mucin immunogens from recombinant vaccinia viruses. Because this virus will replicate in humans, and therefore, it may present a health hazard. Therefore, we have redirected our efforts to expressing the recombinant mucin polypeptide via fowlpox viruses. These viruses are capable of initiating infection of mammalian cells, but the infection is not productive, i.e., the virus does not replicate. Thus, fowlpox viruses have the potential of providing safe vaccines by producing antigens intracellularly (facilitating antigen presentation) when the inserted gene is designed to be expressed prior to replication. We have successfully grown fowlpox virus (not shown) and work is currently in progress to obtain the recombinant mucin cDNA-containing virus.

We have also shown in the first three years of this Career Development Award that the humoral antigenicity of the MUC1 mucin peptides is maximized at 40 amino acids (i.e., two tandem repeats), and therefore does not necessitate a longer, or full length, molecule. By contrast, the cellular immunogenicity of these peptides is somewhat different than the humoral antigenicity. Whereas mucin tandem repeat polypeptides as small a 20 amino acids (one tandem repeat unit) are weakly antigenic against mAb, these peptides elicit the reproducible expansion of tumor-specific CTL from the PBMC of donors with breast cancer. Also, a conservative mutation of a potential glycosylation site in a tumor-specific epitope is effective in eliciting both humoral and cellular immunogenic responses that are tumor-specific; this result makes feasible the ability make similar mutations in other potential glycosylation sites. The expansion of mucin-specific T cells is oligoclonal, and work is in progress to clone these human CTL. Antigen recognition of the expanded CTL at the level of effector function is not HLA-restricted, but may be MHC-dependent for expansion. This stimulation of PBMC with synthetic peptides represents an advance in current methodologies using tumor, or transformed dendritic cells (30) as the immunogen in that an unlimited supply of the synthetic immunogen can be obtained, whereas use of autologous tumor is limited to only the amount of tumor obtained and transformation of dendritic cells is a cumbersome process.

We have continued to make progress in defining the structure immunogenicity relationships of this tumor-specific molecule. Increasing the length of the MUC1 immunogen to two tandem repeats did not enhance the cellular immunogenicity of this peptide. This is consistent with NMR structural studies showing that MUC1-mtr$_1$ and MUC1-mtr$_2$ have predominantly a non-ordered conformation. However, a significant population of peptide conformers may exist in a single tandem repeat to elicit the immunologic response. By

contrast to the single and double tandem repeat peptides the 60 amino acid MUC1-mtr_3 is suggested to have an ordered knob-like conformation (11-13). Consistent with this idea, the MUC1-mtr_3 reproducibly expands tumor-specific CTL 9-fold better than either the single or double tandem repeat peptides. Unexpectedly, our initial NMR structural studies of the MUC1-mtr_3 do not substantiate the earlier reports of Fontenot et al. (11, 13). Work will continue in the last year of this grant to further understand the NMR results in relation to preferred conformation of the polypeptides and immunogenicity. To help resolve the issue of the improved immunogenicity of the MUC1-mtr_3, we have designed and synthesized two additional MUC1-mtr_3 polypeptides. These new peptides have the same molecular mass and amino acid composition as the native MUC1-mtr_3, but one peptide has the first repeat element scrambled, and the other has the second repeat domain scrambled. Thus, these new peptides have the mass of MUC1-mtr_3, but resemble MUC1-mtr_2 and MUC1-mtr_1, respectively. Assay of these peptides plus the MUC1-mtr_5, will help to explain whether the mass of the peptide is important in eliciting a maximal immune response, or whether, a small fraction of the molecules adopt a conformation that is optimal for antigen processing and presentation. Thus, it is critical to the analysis of the structure-immunogenicity relationships of MUC1 mucin peptides that we continue with this structural characterization in the last year of this grant.

We have also proposed a model to describe the arrangement of the oligosaccharide side chains around the core protein. In the remaining year of this Award, we are progressing toward further describing the influence that the length of the core protein has on the structure and immunogenicity of this tumor-specific immunogen and refining the model of mucin.

In conclusion, this work on this Career Development Award is proceeding on schedule with regard to the specific aims and statement of work. Aim 1 and Task 1 have substantially been completed with the synthesis of multiple mucin immunogen peptides and delineation of the parameters of humoral and cellular immunogenicity. Task 2 has been slowed due to the necessary change in recombinant virus expression system, but is well under way and we anticipate that a recombinant virus expressing a MUC1-mucin polypeptide immunogen will be obtained. Task 3a, to determine the recognition of the tumor-specific epitope by mucin-specific mAb has been completed. Task 3b, to describe the cellular immunogenicity of the peptide immunogens, has largely been completed with experiments now directed at addressing the ability of a MUC1-mtr_5 to stimulate the expansion of tumor-specific CTL, and whether mass of the peptide immunogen is important in directing the immune response. Task 3c, to clone a human tumor-specific CTL against MUC1 mucin, has not yet been completed. We, however, demonstrated oligoclonal expansion of tumor-specific CTL and studies are currently in progress to clone these tumor-specific CTL. Completion of Task 4 is on schedule with the initial description of the apparent conformation of the peptide immunogens, and the development of a model describing a patch-work arrangement of the oligosaccharide side chains along the core protein. With regard to structural characterization all peptides and proteins have been characterized by mass spectroscopy and found to be of a singular species. Thus, we can state with certainty that all peptides and proteins were of the highest purity that allows us to make our conclusion about the structure of these immunogens. Work is in progress, and will continue in the last year of this grant, to refine these models. Lastly, Task 5 is to begin preliminary studies to test the efficacy of the CTL for cancer immunotherapy in a SCID/hu breast tumor animal model. With the successful and reproducible expansion of tumor-specific CTL, we are now in the position to begin with this aim several months earlier

than anticipated. We are in the process of purchasing SCID mice to develop the SCID/hu breast tumor model to test the efficacy of the CTL. Since the submission of this proposal, studies have been published describing such a mouse model (31). Thus, we do not anticipate any problems with establishing our tumor model to test the *in vivo* efficacy of the CTL.

We have made considerable progress in the first three years of this grant towards defining the structure-immunogenicity relationships of tumor-specific MUC1 mucin. We look forward to the remaining year of this work to further our understanding of these relationships to further advance our knowledge for the design novel tumor-specific immunogens for the immunotherapy and vaccination against breast adenocarcinoma.

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Appendix.

Breast Mucin Tumor-specific Epitopes for Cancer Immunotherapy

Grant No: DAMD17-94-J-4161

The following material is a listing of the publications and presentations that have resulted from funding from the above referenced Department of the Army grant during the current reporting period:

Publications:

1. Dombrowski, K.E., Brewer, K.A., Maleckar, J.R., Kirley, T., Thomas, J.W. & Kapp, J.A. (1997) Identification and Partial Characterization of the EctoATPase Expressed by B-Lymphocytes. Arch. Biochem. Biophys., 340, 10-18.
2. Russell, B.G., Moddeman, W.E., Birkbeck, J.C., Wright, S.E., Millington, D.S., Stevens, R.D. & Dombrowski, K.E. (1997) Surface Analysis of Human Mucin by X-ray Photoelectron Spectroscopy: A model for the distribution of carbohydrate along the core protein. Biospectroscopy. Accepted with revision.
3. Wright, S.E., Kilinski, L., Talib, S., Lowe, K.E., Dombrowski, K.E., Lebkowski, J.S. & Philip, R. Cytotoxic T-lymphocytes Induced by Native and Glycosylation Site-mutated MUC1 Mucin Peptides from Humans with Adenocarcinomas. Submitted.
4. Dombrowski, K.E., Ke, Y. & Kapp, J.A. (1997) Role of EctoATPase in Lymphocyte Effector Function in EctoATPases: Recent progress in structure and function (Plesner, L., Kirley, T.L. & Knowles, A.F., eds.) Plenum, NY, Ch. 24, pp 197-207.
5. Dombrowski, K.E. & Kapp, J.A. The Role of EctoATPase in Lymphocyte Effector Function. Immunol. Rev. Invited review, submitted to be published Feb. 1998.

Abstracts and Presentations:

1. Dombrowski, K.E. & Kapp, J.A. (1996) Possible Role of EctoATPase in Lymphoid Cell Function. First International Workshop on EctoATPases. Mar del Plata, Argentina, August 26-30.
2. Dombrowski, K.E. & Kapp, J.A. (1996) Role of EctoATPase in the Salvage of Extracellular Nucleotides. First International Workshop on EctoATPases. Mar del Plata, Argentina, August 26-30.
3. Dolby,N., Dombrowski, K.E., & Wright, S.E. (1996) Design and Expression of a Synthetic Mucin Epitope Polypeptide in Escherichia coli. Fourteenth Annual Texas Regional Immunology Conference, San Antonio, TX, November 15-17.
4. Wright, S.E., Lowe, K.E., Talib, S., Kilinski, L., Dombrowski, K.E., Lebkowski, J.S., Philip, R. (1997) Antigen-Specific Cytotoxic T-Lymphocyte (CTL) Response Induced by Tumor-Specific MUC1 Mucin Peptide from Humans with Adenocarcinomas. Keystone Symposum: Cellular Immunology and the Immunotherapy of Cancer - III, Silverthorne, CO. Abstr. #238.